Immuno-detection of *Staphylococcus aureus* Biofilm on a Cochlear Implant

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Abstract

Case presentation: A 46-year-old man suffering from progressive deafness since childhood received a Clarion 90 K cochlear implant with the HiRes® preformed electrode in his left ear in October 2006. A persistent *Staphylococcus aureus* infection failed to be treated with corticoids, amoxicillin/clavulanate, ciprofloxaxin, and rifampin. The processor was removed on July 2007.

Interventions: The removed cochlear implant processor was treated with different reagents, with the aim of detecting a *S. aureus* and *S. aureus* biofilm: (1) fluorescein-coupled Fc of anti-human serum, (2) polyclonal anti-polysaccharide intercellular adhesion antibodies coupled to Alexa Fluor 568 goat anti-rabbit immunoglobulin (Ig)G, (3) crystal violet, (4) methylene blue, (5) acridine orange, (6) Gram stain, and (7) live/dead fluorescent stain.

Results: *S. aureus* and the major constituent of the *S. aureus* biofilm, the polysaccharide intercellular adhesion, were detected on the surface of the implant. *S. aureus* was isolated after a simple contact between the implant and a solid growth medium. The ability of the isolated *S. aureus* strain to produce biofilm *in vitro* was confirmed.

Interpretation: *S. aureus* biofilm was documented on the implant. Initial bacterial colonization could be related to the pocket of the removable magnet. Colonies of *S. aureus* without biofilm were found attached to the electrode wire.

Conclusion: We report one case of a *S. aureus* biofilm infection documented on a cochlear implant, as assessed by immuno-microscopy. The biofilm was likely responsible for the persistent infection which manifested for many months after the implant surgery and could explain the unusual bacterial phenotypic resistance against administered antimicrobial agents.

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Introduction

Until recently, medical complications associated with cochlear implants were mainly related to flap necrosis, incision dehiscence, and post-operative wound infections [4, 11, 23]. The incidence of such complications was low, and they were usually successfully managed with antibiotics and/or plastic and middle ear surgery. Cases of persistent infection requiring the removal of the processor have been rare, and some have even been attributed to a primary immunodeficiency of the recipient [23]. Similarly to what has been observed on other types of implants [5], reports of biofilm formation on the surface of cochlear implants started to appear in print in 2004 [1, 17, 20]. Clinically, biofilms are complex bacterial communities that adhere to the surface of implanted biomaterial or mucosa [9, 18] and produce an extra-cellular matrix [3], leading to increased bacterial resistance against the host's immune defenses and to antibiotics [22]. Since 1985, 200 patients have been implanted at the Geneva Cochlear Implant Centre. Different types of implants have been used and, until recently, not a single case of wound dehiscence, flap necrosis, or infection of the processor has been observed. In 2007, we were confronted with an atypical and persistent infectious case that required removal of the processor, a Clarion 90 K cochlear implant with the HiRes® preformed electrode, which was then submitted to microbiological analysis.

Case History

In October 2005, a 46-year-old man suffering from progressive deafness since childhood received a Clarion 90 K cochlear implant with the HiRes® preformed electrode in his left ear.

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Surgery was performed according to the minimally invasive technique described by*O'Donoghue and Nikolopoulos* [19]. The processor was placed in a muscle pouch and attached to the bone. The patient was given intravenous ceftriaxone during the surgery, followed by amoxicillin/clavulanate (orally) for 1 week post-surgery. Healing was uneventful. Two months after cochlear implantation the patient had achieved a very good performance and was wearing his implant daily. 17 months after the surgery, however, he felt an increasing retro-auricular pain, and the site of the processor became swollen. We suspected that a hematoma had developed inside the muscle pouch containing the processor and treated the symptoms with corticoids and amoxicillin/clavulanate orally. The swelling and pain disappeared in 15 days, but reappeared 6 weeks later. Puncture removed 3 cc of a citrin liquid, and subsequent culture of the liquid showed the growth of *Staphylococcus aureus* sensitive to all tested antibiotics with the exception of penicillin G. The patient received a treatment of ciprofloxacin and rifampin for 8 weeks, but the swelling, redness, and pain persisted. Surgical drainage was performed. The surrounding tissue was debrided, and the muscle pouch and the processor were irrigated with antibiotics. Culture showed again *S. aureus*. The wound healed, but 2 weeks later liquid had again collected. The processor was removed in July 2007. The electrode wire was sectioned at the level of the cochleostomy, and the electrode array was left inside the cochlea. The wound healed in 2 days. Three months later the patient received a new cochlear implant, which was switched on 2 weeks after surgery. The patient has since reached the same performance levels with the new implant as he had with the original one.

Material and Methods

Microbiological Sampling, Cultures, and Identification of the Strain of *S. aureus*

During the removal surgery, samples were collected from the inguinal, axillary, retro-auricular, and external ear canal skin, from nasal and throat mucosa, and from fragments of the surrounding tissue. The identification of a strain of *S. aureus* was performed according to Clinical and Laboratory Standards Institute (CLSI) recommendations and included Pastorex agglutination (Bio-Rad, Hercules, CA) and the DNAse production test. A real time-PCR amplification procedure [8] was performed for confirmation.

S. aureus growth was achieved by culturing the removed processor on Mueller Hinton agar (MHA; Bio-Rad, Marnes-La-Coquette, France). *S. aureus* strain SA113 (ATCC 35556) and its *ica* mutant (*Δica::tet*) were used as control for immuno-detection of polysaccharide intercellular adhesion (PIA) [10]. MHA and trypticase soy broth (TSB; Becton Dickinson, Le Pont de Claix, France) supplemented with 1% glucose (TSBgluc) were used for bacterial growth. The *in vitro* formation of the biofilm was tested on a strain grown in TSBgluc medium during a 15-h culture. A glass coverslip was added to the well prior to culture for the detection of PIA.

Pre-Treatment of the Processor

The processor body was divided into two parts. The silicon part harboring the removable magnet pocket after the magnet was removed was snap-frozen in liquid nitrogen and conserved at 80 °C. Before testing, the device was washed with a PBS solution (Invitrogen, Carlsbad, CA) and sliced into five pieces, which were deposited in separated wells on a six-well plate. The other part, which included the electrode wire, was washed with PBS, immediately fixed for 1 h in 2% v/v glutaraldehyde (Fluka, Chemica, Germany) in PBS, and conserved in a PBS solution at 4 °C. Before testing, this part and the electrode wire were sliced into five pieces each.

Crystal Violet Staining Assay for Initial Evaluation of the Presence of the Biofilm

Glutaraldehyde-fixed implant sections and the*in vitro* heat-fixed bacteria biofilm were stained for 10 min with 1% (w/v) crystal violet (CV) stain freshly diluted twofold in 1% ethanol/distilled water, as previously described [21]. The stained material was then washed three times with PBS and inspected with the naked eye and by white light microscopy.

Immuno-Detection of PIA

Sections of the processor and *in vitro* biofilm from the *S. aureus* grown on a circular glass coverslip (diameter 25 mm) were washed twice with PBSAT (PBS containing 0.02% azide and 0.05% Tween. 20) with slow shaking for 5 min. Cross-reactions with *S. aureus* protein A were blocked by incubating the material for 2 h with 1:1,000 normal donkey serum (Jackson Immuno Research, West Grove, PA). For specific detection of PIA, the material was incubated for 1 h with 1:3,000 α-PIA rabbit polyclonal anti-PIA antibody [15], then washed twice with PBSAT. The binding of specific antibodies was revealed after incubation with 1:3,000 dilution of Alexa Fluor 568 goat anti-rabbit IgG (H + L) (Molecular Probes, Eugene, OR) as secondary antibody. The material was washed with a PBS solution (PBSAT) containing 1% albumin (ZLB Behring AG, Bern, Switzerland) and 0.1% tween-20 (Fluka).

Immuno-detection of *S. aureus* Through Binding to Cell-Wall Protein A

Processor sections and the slices of the electrode wire were incubated for 30 min with PBSAT and for 30 min with 1:1,000 fluorescein-coupled to the Fc fragment of goat anti-human serum (Jackson Immuno Research). The slices were then washed five times with PBSAT before being observed under the microscope.

Immuno-Fluorescent Microscopy

All incubations were performed in PBSAT. A humid chamber was prepared for antibody incubations, consisting of a six-well plate that was hermetically sealed with Parafilm and light-protected with aluminium foil. Images were acquired by an Axiocam color camera (Zeiss, Iena, Germany) on an Axioskop 2 microscope (Zeiss). Ultraviolet excitation for fluorescent imaging and white light microscopy were performed both separately and in combination. Filter set 09 (Zeiss; excitation BP 450–490, emission LP 515) was used for PIA immuno-detection through Alexa Fluor detection (emission 603 nm), whereas Filter set 02 (Zeiss; excitation G 365, emission LP 420) was used to detect fluorescein isothiocyanate (FITC; emission 530 mm ± 15 nm), indicating the presence of *S. aureus* through the binding of the Fc fragments to bacterial protein A. Scaling was performed automatically with the AxioVision software (Zeiss) according to the objective in use.

Results

Bacterial Recording

Staphylococcus aureus was identified in the external ear canal and retro-auricular swabs, in the tissue fragments, and in the liquid surrounding the implant collected during the removal surgery. Identification was performed

according to CLSI recommendations. Identification of the bacterium was based on a positive by Pastorex agglutination and DNAse production tests. Confirmation was obtained using a previously described duplex PCR amplification. *S. aureus* was also detected by the direct growth of the bacterium following culture of the explanted implant on a solid medium in a petri dish, yielding a pure culture. The isolated *S. aureus* strain was named ''Coch''.

S. aureus Biofilm Identification on the Cochlear Implant

The biofilm was detected on the glutaraldehyde-fixed part of the implant containing the magnet pocket (Figure 1a). Cochlear implant pieces were successfully stained with CV in different zones visible to the naked eye. These colored zones were less abundant than areas reacting with the methylene blue stain. White-light microscopic observations of CV-stained zones showed an association with cocci (Ø = 1 μm) (Figure 1b). Presence of adherent *S. aureus* on the implant was confirmed by the immunodetection of protein A using a FITC-coupled immunoglobulin (Figure 1c). The presence of the biofilm was postulated in these CV-stained zones and confirmed using specific antibodies raised against *S. aureus* PIA (Figure 1d). Surface PIA-positive zones were less abundant than CV-stained zones.

In vitro PIA-based Biofilm Formation

Coch strain produced PIA *in vitro* on glass coverslips at amounts quite similar to those of the control laboratory strain SA113 (Figure 2). Surface colonization to be appeared homogeneous for strain SA113 *in vitro*, whereas strain Coch showed punctual aggregates. The *ica* mutant was negative for PIA-specific fluorescence (Figures 1, 2).

Binding of Fluorescent Fc Fragments on the Electrode Wire

Immuno-detection performed on the electrode wire was positive for *S. aureus* on the first five proximal sections (corresponding to a 4.4-cm length from the implant side) as revealed by Fc-FITC binding (not shown). The three most distal fragments studied were negative.

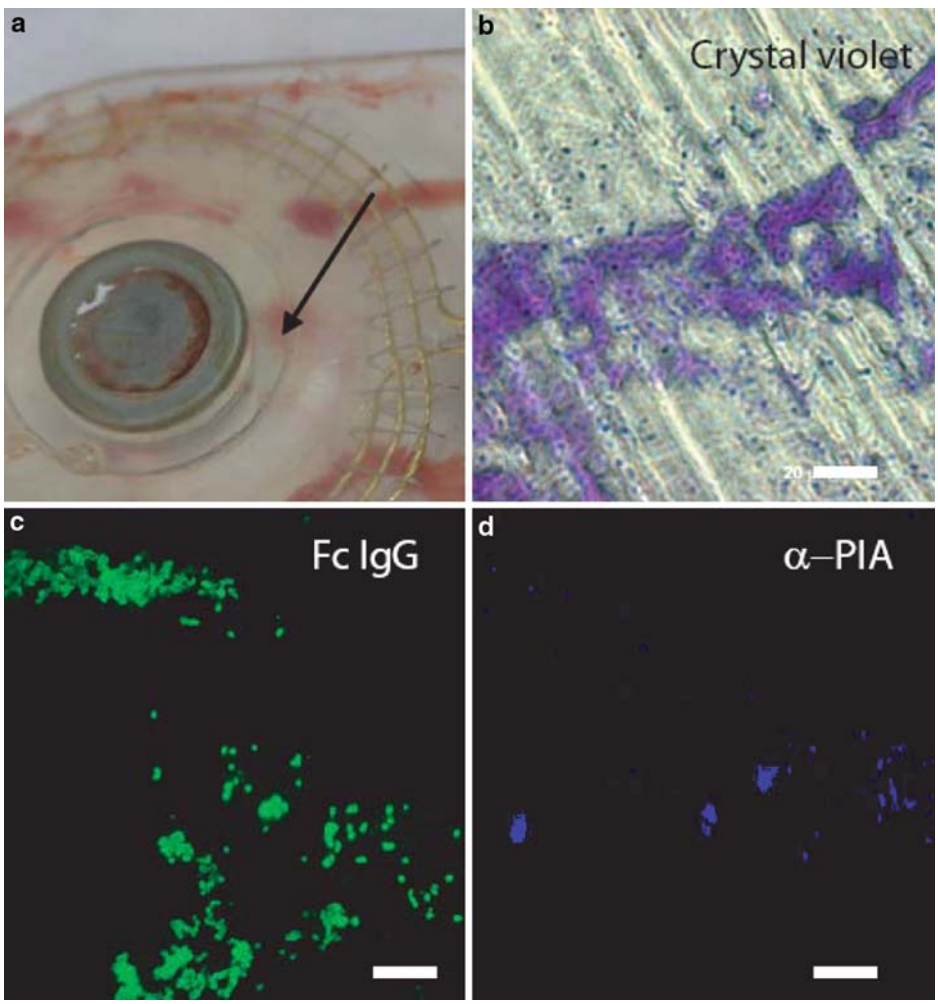

Figure 1. Microscopy imaging of the removed cochlear implant. a) Magnet pocket of the explanted Clarion 90 K cochlear implant. The successfully stained zones depicted on panels b, c, d are localized near the recess surrounding the magnet (Ø = 8 mm, see arrow). b) Crystal violet-stained zone associated with cocci-like structures. c) Immunofluorescence microscopy showing the presence of *S. aureus* protein A. d) Immuno-fluorescence microscopy showing the presence of biofilm-associated polysaccharide intercellular adhesion (PIA). Scale bars: 20 μm.

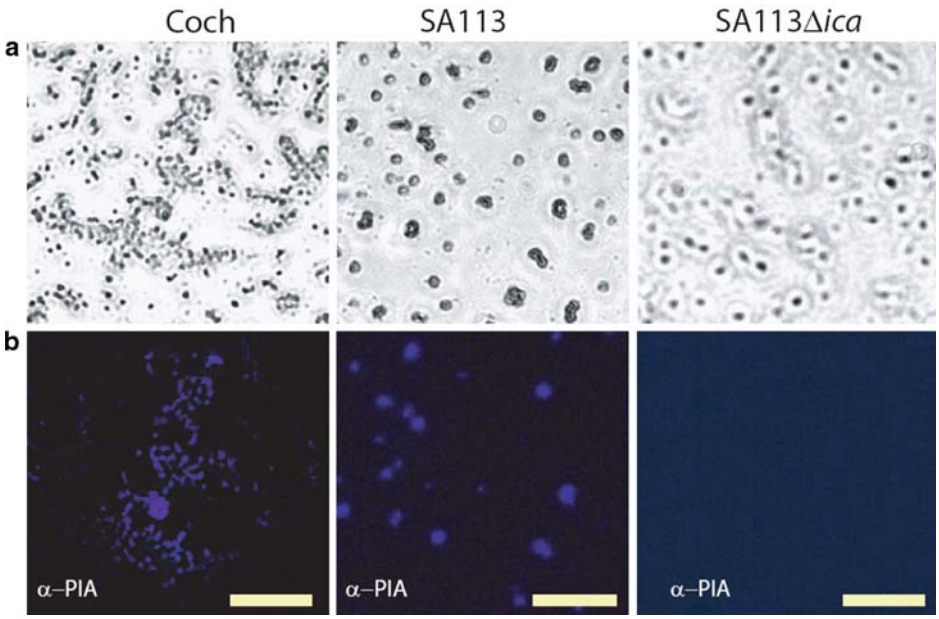

Figure 2. *In vitro* production of PIA. Microscopic pictures of *in vitro* biofilms formed on glass coverslips of Coch (left column), SA113 (middle column), and SA113*Δica* (right column). Row A: Adherent bacteria visible using white light microscopy. Row B: Pictures of PIA immuno-detection on the corresponding surfaces. Scale bars: 20 μm.

Discussion

Worldwide, only two cases of implant extrusion due to persistent infections by *S. aureus* have been clearly associated to biofilms. In these cases, identification was performed using scanning electron microscopy [1, 20]. We report here a third case of *S. aureus* biofilm infection of a Clarion HiRes 90 K cochlear implant. The microscopic appearance of the biofilm-related PIA matrix produced by the isolated *S. aureus* strain, denoted here as Coch, differed between the *in vitro* and *ex vivo* experiments. CV staining is an easy indirect procedure that can be used to quantify biofilm *in vitro* [7, 21], but it has never been used for *ex vivo* biofilm detection. Our case is therefore the first time this procedure has been applied to detect bacterial biofilm on *ex vivo* materials as an initial evaluation of the surface of the implant. Cocci-like structures (Φ approx. 1 μm) were clearly visible in the microscopic observations. Taken together, PIA immuno-detection and CV staining confirmed the presence of a *S. aureus* biofilm on the surface of the implant inside the pocket of the removable magnet. Analysis of the magnet itself was impossible for technical reasons. We speculate that *S. aureus* contaminated first a hematoma inside the muscle pouch made to hold the processor and subsequently the processor itself. Even though a treatment of amoxicillin/clavulanate followed by ciprofloxacin and rifampin was given as soon as a biofilm infection was suspected, the processor had to be ultimately explanted. This case illustrates how efficiently the bacterial community resisted the host immune response and the antibiotics that are effective when the same bacteria are in their planktonic form [3] and confirms that biofilm contamination of an implant often requires removal of the latter [5]. *S. aureus* had already been detected on the culture of the puncture carried out at the early stage of the disorder. There have been published cases of redness and tenderness around the processor followed by flap necrosis or dehiscence and, ultimately, rejection of the processor. These cases were suspected to be caused by an allergy to one of the implant components [16] because no germs could be detected. However, even in such cases, biofilm infections should not be excluded. Sub-clinical biofilm infections can persist for many years before they manifest [11]; therefore, a biofilm infection cannot be ruled out based on a negative culture of the material [14].

Antibiotic prophylaxis is recommended for surgeries involving implants, but the postoperative use of antibiotics is not [2]. This latter practice has, since this case, been abandoned by our center. Although we consider the case reported here to be unique among all the procedures carried out at our center, representing 0.5% of our implants, the post-operative use of antibiotics could have contributed to increasing the resistance of the infecting bacteria and the formation of biofilm.

The first processors implanted in the 1980s were sealed in a smooth ceramic case [13]. The contribution of bacterial biofilm-related infection to the frequency of implant removal is clearly under-reported in the literature as biofilm presence has not been not systematically assessed. In 2000, new processors constructed out of flexible silastic and containing a removable magnet pocket were developed by Cochlear® and by Advanced Bionics®. Two recent studies in which this type of implant was used have reported the presence of biofilm scattered over the entire the surface of the devices [1, 20]. The authors of another report observed a higher abundance of biofilm and biofilm-associated bacteria inside the depressions on the body processor [17]. In the latter study, *Loeffler* et al. [17]

also reported higher counts of bacteria on implants harboring an empty magnet pocket as compared to models without a magnet pocket.

As reported previously by other authors [1, 17, 20], we cannot confirm the presence of biofilm around the electrode wire. The tests performed after the removal of the processor revealed that there was *S. aureus* attached to the electrode wire up 4.4 cm distally from the body of the processor, but these are not specific for the detection of biofilm. In our case, during the removal surgery, the length of the wire that extended from the mastoid to the cochleostomy appeared to be normal. We arbitrarily decided to cut the wire at the level of the cochleostomy and leave the electrodes array inside the cochlea to avoid obliteration by fibrous tissue that could prevent a new implant.

Modern processors equipped with a removable magnet represent a major improvement for patients suffering from chronic disease that requires regular follow-up with MRIs. The ability to remove the magnet removes the risk of magnet mobilization or demagnetization in patients requiring radiological examination. Unfortunately the pocket designed to encase the magnet seems to favor biofilm formation, and the possibility that this pocket is involved in biofilm formation should not be ignored. Technical developments are under way to avoid the formation of bacterial biofilms, such as surface treatment with antimicrobial molecules [6]. Clearly, such developments need careful evaluation in the clinical context [6, 12].

In conclusion, biofilm can cause resistant infections of cochlear implants that manifest many months after the surgery. The pocket of the removable magnet could be one niche facilitating biofilm, formation although colonization of the electrode wire is not excluded.

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Reference

1. Antonelli PJ, Lee JC, Burne RA: Bacterial biofilms may contribute to persistent cochlear implant infection. Otol Neurotol 2004; 25: 953–957.
2. Bratzler DW, Houck PM: Antimicrobial prophylaxis for surgery: an advisory statement from the National Surgical Infection Prevention Project. Am J Surg 2005; 189: 395–404.
3. Costerton JW, Stewart PS, Greenberg EP: Bacterial biofilms: a common cause of persistent infections. Science 1999; 284: 1318–1322.
4. Cunningham CD III, Slattery WH III, Luxford WM: Postoperative infection in cochlear implant patients. Otolaryngol Head Neck Surg 2004; 131: 109–114.
5. Darouiche RO: Treatment of infections associated with surgical implants. N Engl J Med 2004; 350: 1422–1429.
6. Darouiche RO: Antimicrobial coating of devices for prevention of infection: principles and protection. Int J Artif Organs 2007; 30: 820–827.
7. Djordjevic D, Wiedmann M, McLandsborough LA: Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl Environ Microbiol 2002; 68: 2950–2958.
8. Francois P, Bento M, Renzi G, Harbarth S, Pittet D, Schrenzel J: Evaluation of three molecular assays for rapid identification of methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol 2007; 45: 2011–2013.
9. Galli J, Calo L, Ardito F, Imperiali M, Bassotti E, Fadda G, Paludetti G: Biofilm formation by *Haemophilus influenzae* isolated from adeno-tonsil tissue samples, and its role in recurrent adenotonsillitis. Acta Otorhinolaryngol Ital 2007; 27: 134–138.
10. Gotz F: *Staphylococcus* and biofilms. Mol Microbiol 2002; 43: 1367–1378.
11. Harada T, Ishida K, Endo M, Takahashi M, Sakai M: Recurrent extrusion of cochlear implant at an interval of 5 years. Otol Neurotol 2003; 24: 83–85.
12. Johnson TA, Loeffler KA, Burne RA, Jolly CN, Antonelli PJ: Biofilm formation in cochlear implants with cochlear drug delivery channels in an in vitro model. Otolaryngol Head Neck Surg 2007; 136: 577–582.
13. Kessler DK: The CLARION multi-strategy cochlear implant. Ann Otol Rhinol Laryngol Suppl 1999; 177: 8–16.
14. Klykken PC, Curtis JM: Re: "Silicone allergy: a new cause for cochlear implant extrusion and its management". Otol Neurotol 2007; 28: 1159–1161.
15. Knobloch JK, Horstkotte MA, Rohde H, Mack D: Evaluation of different detection methods of biofilm formation in *Staphylococcus aureus*. Med Microbiol Immunol 2002; 191: 101–106.
16. Kunda LD, Stidham KR, Inserra MM, Roland PS, Franklin D, Roberson JB Jr: Silicone allergy: a new cause for cochlear implant extrusion and its management. Otol Neurotol 2006; 27: 1078–1082.
17. Loeffler KA, Johnson TA, Burne RA, Antonelli PJ: Biofilm formation in an in vitro model of cochlear implants with removable magnets. Otolaryngol Head Neck Surg 2007; 136: 583–588.
18. Morris DP, Hagr A: Biofilm: why the sudden interest? J Otolaryngol 2005; 34: S56–S59.
19. O'Donoghue GM, Nikolopoulos TP: Minimal access surgery for pediatric cochlear implantation. Otol Neurotol 2002; 23: 891–894.
20. Pawlowski KS, Wawro D, Roland PS: Bacterial biofilm formation on a human cochlear implant. Otol Neurotol 2005; 26: 972–975.
21. Tu Quoc PH, Genevaux P, Pajunen M, Savilahti H, Georgopoulos C, Schrenzel J, Kelley WL: Isolation and characterization of biofilm formation-defective mutants of *Staphylococcus aureus*. Infect Immun 2007; 75: 1079–1088.
22. Vlastarakos PV, Nikolopoulos TP, Maragoudakis P, Tzagaroulakis A, Ferekidis E: Biofilms in ear, nose, and throat infections: how important are they? Laryngoscope 2007; 117: 668–673.
23. Yu KC, Hegarty JL, Gantz BJ, Lalwani AK: Conservative management of infections in cochlear implant recipients. Otolaryngol Head Neck Surg 2001; 125: 66–70.